CERTIFICATION

I, Hiroaki ITO, whose address is Fujita-Toyobo Building 9th floor, 1-16, Dojima 2-chome, Kita-ku, Osaka-shi, Osaka, JAPAN, hereby certify that I am the translator of the attached document, namely,

Japanese Patent Application No. 2003-113707

that I am familiar with both the Japanese language and the English languages, and that the translation is a true and correct translation from the Japanese language to the English language to the best of my knowledge and belief.

This 9th day of August, 2006

Hiroaki ITO

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[Title of the Invention] METHOD FOR LABELING PHOSPHORYLATED PEPTIDES, COMPLEX COMPOUNDS USED IN THE METHOD, PROCESS FOR PRODUCING THE COMPLEX COMPOUNDS, AND RAW MATERIAL COMPOUNDS FOR THE COMPLEX COMPOUNDS

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[List of Deliverables]

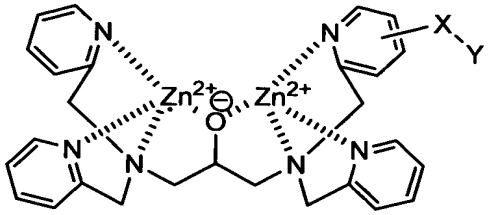
[Name of Object]	Specification	1
[Name of Object]	Drawing	1
[Name of Object]	Abstract	1
[Number of General Power of Attorney]		9709342

[Document] Description

[Title of the Invention] METHOD FOR LABELING PHOSPHORYLATED PEPTIDES, COMPLEX COMPOUNDS USED IN THE METHOD, PROCESS FOR PRODUCING THE COMPLEX COMPOUNDS, AND RAW MATERIAL COMPOUNDS FOR THE COMPLEX COMPOUNDS

[Claims]

[Claim 1] A method for labeling a phosphorylated peptide by a complex compound represented by the formula (I):

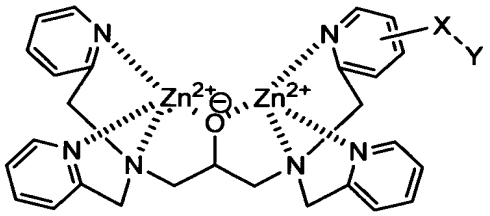


(I)

wherein X is a linker moiety, and Y is a labeling group.

[Claim 2] The method according to Claim 1, wherein the complex compound is a compound having biotin as the labeling group.

[Claim 3] A complex compound represented by the formula (I):



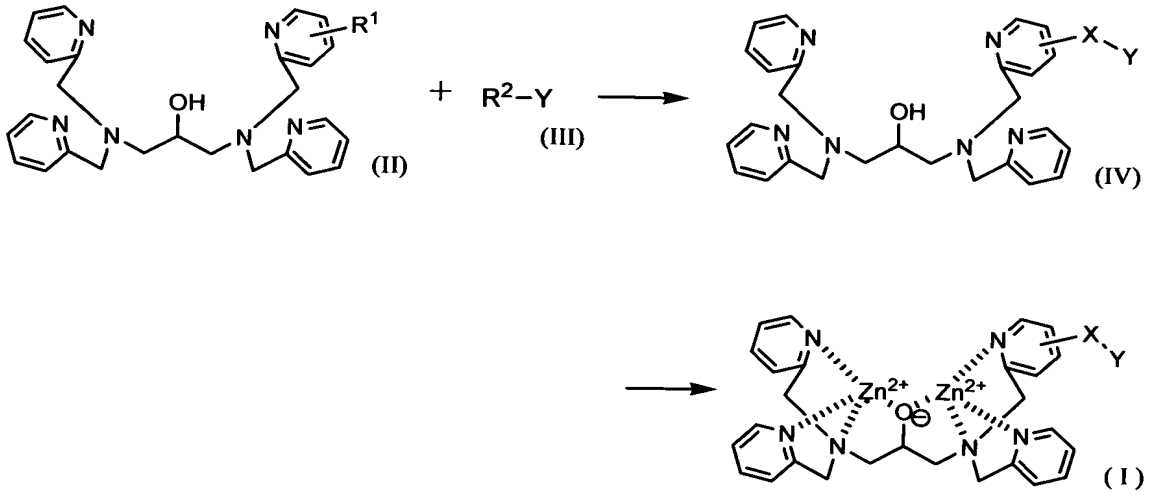
(I)

wherein X is a linker moiety, and Y is a labeling group.

[Claim 4] The complex compound according to Claim 3, wherein the labeling group is biotin.

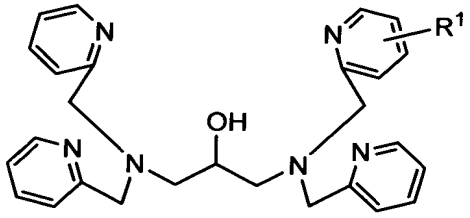
[Claim 5] A method for producing the compound (I), comprising Scheme 1.

Scheme 1



wherein R^1 and R^2 each is a reactive group for forming the linker moiety X, and Y is a labeling group.

[Claim 6] A compound represented by the formula (II):



(II)

wherein R^1 is a reactive group except an aminomethyl group, a hydroxymethyl group, an amino group, and a carboxyl group.

[Detailed Description of the Invention]

[0001]

[Technical Field of the Invention]

The present invention relates to a method for labeling phosphorylated peptides, complex compounds usable in the methods, a process for producing the complex compounds, and compounds usable as a raw material in the production process.

[0002]

[Prior Art]

There are known in vivo enzymes having serine, threonine or tyrosine residue at a specific site corresponding to an active center. The enzymatic activity of these enzymes is

controlled by phosphorylating or dephosphorylating hydroxyl group in these residues by an enzyme called kinase. Also, there are known enzymes whose enzymatic activity is controlled by phosphorylating or dephosphorylating an nitrogen atom in lysin, arginine or histidine, or a carboxyl group in aspartic acids or glutamic acids.

[0003]

One of the examples of the metabolic systems which are controlled by the aforementioned phosphorylation-dephosphorylation is a system of suppressing synthesis of glycogen and decomposing the same. This metabolic system is primarily cascade-controlled by the phosphorylation-dephosphorylation.

[0004]

A recent study elucidated that the phosphorylation-dephosphorylation plays a significant role in disease-related metabolic systems.

[0005]

For instance, it is said that one of the causes of cell carcinogenesis is abnormality in the phosphorylation-dephosphorylation. Specifically, progress and stop of cell cycle are controlled by phosphorylation or dephosphorylation of various enzymes, i.e., proteins. Cycline and cycline-dependent kinase (CDK) are relevant factors in the phosphorylation. If the mechanism relating to cycline and CDK is impaired, phosphorylation or dephosphorylation may be uncontrollable, thereby triggering abnormal proliferation of cells.

[0006]

In addition to the above, facts are known that protein

kinase C is related with degranulation of histamine causative of allergic disorders such as atopic dermatitis and pollen allergy, and that phosphorylated tau-protein is causative of neurofibrillary tangle in the brains of Alzheimer's patients.

[0007]

In view of the above, comprehending the condition of phosphorylation-dephosphorylation of proteins provides useful measures not only in investigating expression of genes in living tissue cells and evaluating the enzymatic activity of the cells, but also in diagnosing diseases or medical treatment.

[0008]

The conventional methods for identifying phosphorylated proteins or dephosphorylated proteins have various drawbacks.

[0009]

For instance, while an enzyme immunoassay is advantageous in analyzing a target protein sample of a very small amount, it is difficult to obtain antibodies of the target protein of a sufficient amount. Further, in case that the level of the target protein is several kDa or lower, it is impossible to prepare an antibody that is securely bonded to a site in the protein where phosphorylation occurs.

[0010]

There is proposed a method for detecting a protein specifically bonded by a phosphoric acid with use of a phosphoric acid labeled with a radioactive isotope ^{32}P. However, special attention should be paid in handling radioactive isotopes, and appropriate administration and disposal of waste liquid of the radioactive isotopes are required.

[0011]

There is proposed an idea of applying two-dimensional

electrophoresis in view of the fact that electric charges are differentiated between phosphorylated proteins and dephosphorylated proteins. However, it is extremely difficult to identify the band or spot of a phosphorylated or dephosphorylated protein in analyzing a sample derived from a living organism, because the sample contains a variety of proteins. Furthermore, use of a radioactive isotope to identify the band or spot involves the aforementioned problems.

[0012]

The non-patent document 1 recites a zinc complex. The zinc complex has a function that two zinc ions in the complex dissociate a phosphoric acid group from dinucleotide. However, the function of the zinc complex disclosed in the document is merely a catalyst. The non-patent document 1 dose not disclose the ability of the zinc complex to bond coordinately to a phosphoric acid group. The experiments conducted by the inventors reveal that a dissociation constant of the zinc complex to a phosphoric acid group sandwiched by two nucleosides, namely, a phosphoric diester, is extremely high. In other words, the zinc complex has a low coordinatability to a phosphoric diester moiety.

[0013]

Further, the non-patent document 2 recites an iron complex having a structure analogous to the structure of the zinc complex. The iron complex, however, is a product synthesized as a model of hemerythrin, namely, a carrier protein carrying oxygen molecules. As is the case with the above non-patent document 1, this document neither discloses nor remotely suggests coordinate bond of the iron complex to a phosphoric group.

[0014]

[non-patent document 1]

Morio YASHIRO, and two members, 「Preparation and Study of Dinuclear Zinc(II) Complex for the Efficient Hydrolysis of the Phosphodiester Linkage in a Diribonucleotide」, Journal of the Chemical Society, Chemical communications, pp.1793-1794 (1995)

[non-patent document 2]

Hidekazu ARII, and six members, 「A novel diiron complex as a functional model for hemerythrin」, Journal of Inorganic Biochemistry, 82, pp.153-162 (2000)

[0015]

[Problems to be Solved by the Invention]

In view of the above, it is an object of the present invention to provide a method for labeling phosphorylated peptides, namely proteins, for easy detection.

[0016]

It is still another object of the present invention to provide compounds that are capable of being highly coordinated to the phosphorylated peptides and usable in the labeling method, a process for producing the compounds, and raw material compounds usable in the production process.

[0017]

[Means for Solving the Problems]

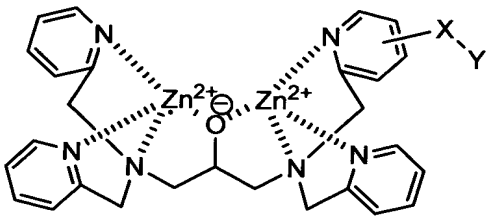
For solving the above-mentioned problem, the present inventors have intensively studied a metal complex which is capable of bonding to phosphoric group. As the result, the present inventors found that the inventive compound has very high coordinate linkage ability to the phosphoric group, i.e. phosphoric monoester group, bonded to peptide. Therefore, the

inventive compound can selectively bond phosphorylated peptide to form complex even in mixed sample containing a number of peptides. Further, since the inventive compound has a labeling group, the complex can easily be identified. Accordingly, the present inventors completed the invention.

[0018]

According to an aspect of the present invention, provided is a method for labeling a phosphorylated peptide by a complex compound represented by the formula (I):

[0019]



(I)

wherein X is a linker moiety, and Y is a labeling

[0020]

Accordingly, the complex compound represented by the above formula (I) can specifically bond to phosphorylated peptide to form a complex, and the complex can be easily identified because of its labeling group. Therefore, the complex compound of the present invention is very useful for study of biochemistry and diagnosing diseases, especially.

[0021]

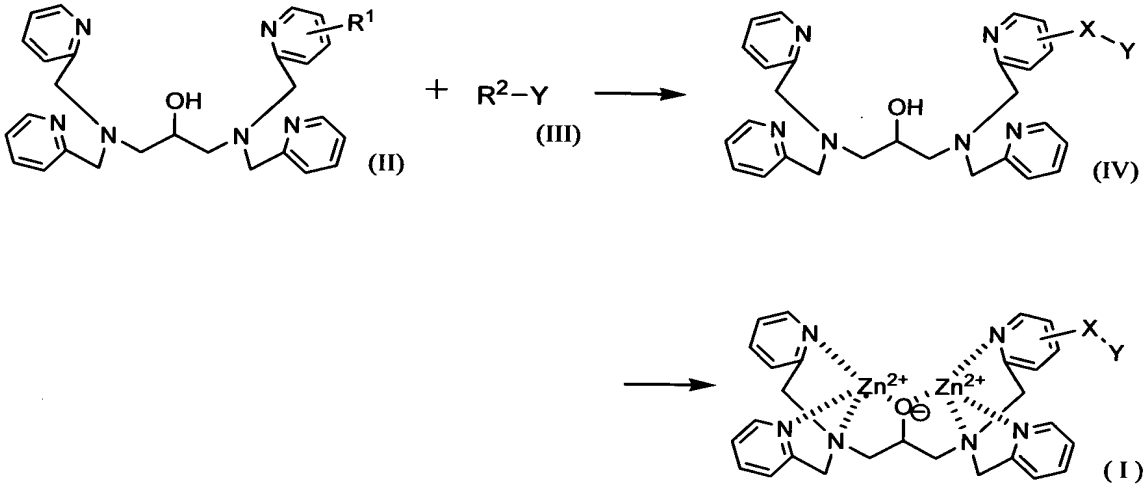
In the above methods, it is preferable to use the compound having biotin as the labeling group. Use of biotin is preferred because biotin is easy to be handled, and has high usability in the point that it exhibits various coloring reactions. Use of biotin is effective in easily identifying the phosphorylated peptide.

[0022]

The complex compound represented by the above formula (I) can be produced by the method comprising Scheme 1.

Scheme 1

[0023]



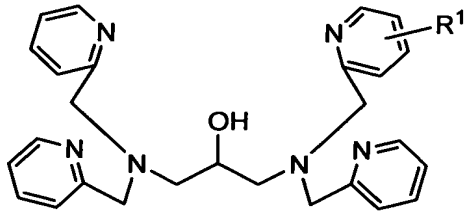
[0024]

wherein R^1 and R^2 each is a reactive group for forming the linker moiety X, and Y is a labeling group.

[0025]

The compound represented by formula (II) is useful as the compound usable in the above scheme 1, namely as the raw material compound of the complex compound represented by the above formula (I).

[0026]



(II)

[0027]

wherein R^1 is a reactive group except an aminomethyl group, a hydroxymethyl group, an amino group, and a carboxyl group.

[0028]

[Mode for Carrying out the Invention]

A primary feature of the inventive method resides in that a phosphorylated peptide can be easily identified by forming a composite compound in which a complex compound having a labeling group as represented by the formula (I) is specifically bonded to the phosphorylated peptide.

[0029]

There have been known various metallic complexes capable of being bonded to a phosphoric acid group. However, a compound which is analogous to the compound represented by the formula (I) and has a labeling group has been unknown. The inventors found that use of the complex compound represented by the formula (I) is advantageous in easily detecting and identifying a phosphorylated peptide even in a sample of a living organism containing a multitude of kinds of peptides, and accomplished the present invention.

[0030]

In the following, a method for labeling phosphorylated peptides according to an embodiment of the present invention is described.

[0031]

First, prepared is a sample containing substantially all the possible kinds of peptides constituting a tissue cell to be examined. The preparation can be conducted according to a conventional method practiced in the biochemistry.

[0032]

Next, the peptides contained in the sample are separated. The separation method is not specifically limited, and a conventional separation method such as electrophoresis can be applied.

[0033]

In case of implementing the electrophoresis, the gel after the electrophoresis is immersed in a solution containing the complex compound represented by the formula (I) to label the phosphorylated peptide, and then, the phosphorylated peptide is detected by a detecting method depending on the kind of the labeling group.

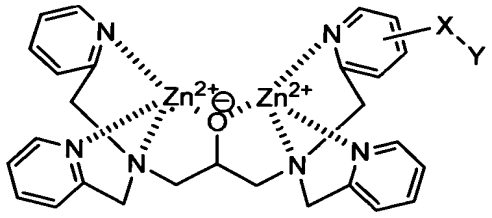
[0034]

A solvent usable in the solution containing the complex compound represented by the formula (I) is not specifically limited, as far as the solvent does not hinder detection of the phosphorylated peptide. Water including a buffer and a solution containing a salt other than a buffer; alcohols such as methanol and ethanol; and a mixed solvent containing these components are examples of the solvent. Preferably, an aqueous solvent is used primarily for the purpose of preventing denaturation of the peptide.

[0035]

Next, the compound (I) used in the above method is described.

[0036]



(I)

[0037]

wherein X is a linker moiety, and Y is a labeling group.

In the formula (I), Zn is selected as a coordinate metal because Zn is highly coordinated to a mono phosphoric acid group in a phosphorylated protein.

[0038]

The linker moiety in the present specification and the claims means a group capable of linking a main skeleton and a labeling group.

The linker moiety facilitates production of the compound (I), and inhibits the labeling group from hindering coordination of the compound (I) to the phosphoric acid group bonded to the peptide. In view of this, the linker moiety may be a coordination bond which directly links the main skeleton and the labeling group if it is easy to obtain a raw material compound in which a labeling group is directly linked to a main skeleton in synthesizing the compound (I), or if the labeling group is of such a small size that hindrance of coordination of the compound (I) to the phosphoric acid group is securely prevented.

[0039]

The kind of the linker moiety in the present specification and the claims is not specifically limited, as far as the linker moiety has the above functions. Examples of the linker moiety are: a C1-C6 alkylene group, an amino group (-NH-), an ether group (-O-), a thioether group (-S-), a carbonyl group (-C(=O)-), a thionyl group (-C(=S)-), an ester group, an amide group, a urea group (-NHC(=O)NH-), a thiourea group (-NHC(=S)NH-); a C1-C6 alkylene group having, at one end thereof, a group selected from the group consisting of an amino group, an ether group, a thioether group, a carbonyl group, a thionyl group, an ester group, an amide group, a urea group, and a thiourea group; and a C1-C6 alkylene group having, at the opposite ends thereof, two groups selected from the group consisting of an amino group, an ether group, a thioether group, a carbonyl group, a thionyl group, an ester group, an amide group, a urea group, and a thiourea group, wherein the groups at the opposite ends are identical to or different from each other.

[0040]

The C1-C6 alkylene group means a bivalent aliphathic hydrocarbon group having 1 to 6 carbon atoms of a straight chain

or a branched chain, such as methylene, ethylene, propylene, tetramethylene, hexamethylene, methylethylene, methylpropylene, and dimethylpropylene, and preferably is a C1-C4 alkylene group, and more preferably is a C1-C2 alkylene group. [0041]

The labeling group in the present specification and the claims is not specifically limited, as far as it is generally used in the biochemistry. However, a compound containing a radioisotope is not preferable in the aspect of handling. Examples of the labeling group are a fluorescent group, a group containing a nitro oxide radical and biotin.

[0042]

The fluorescent group is a substituent capable of stably generating fluorescence of a relatively long wavelength, and a group generally used in the biochemistry can be unlimitedly used as the fluorescent group, irrespective of the property that the compound is soluble in water or oil. Examples of the fluorescent group are aminomethylcoumarin derivatives, fluoroscein derivatives, tetramethylrhodamine derivatives, anthraniloyl derivatives, nitrobenzoxadiazole derivatives, and dimethylaminonaphthalene derivatives.

[0043]

The group containing a nitro oxide radical is a group having a stable radical, and is capable of detecting a phosphorylated peptide by electron spin resonance (ESR). Generally, a biological molecule does not show electron spin resonance because it does not have an unpaired electron. On the other hand, since a peptide coordinately bonded by the compound (I) shows electron spin resonance, the phosphorylated peptide is identifiable.

[0044]

Biotin has specific and high affinity to avidin derived from

albumen and streptoavidin derived from actinomycetes. In view of this, the inventive complex compound can be specifically bonded to an enzyme by way of biotin and avidin or streptoavidin, by bonding avidin or streptoavidin to the compound having biotin as a labeling group and further bonding a biotinated enzyme. The phosphorylated peptide can be identified by using the enzyme such as alkaliphosphotase, peroxidase and luciferase, and by using a coloring reagent depending on the kind of the enzyme. For instance, if alkaliphosphotase is bonded to as the enzyme, further nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as the coloring reagents are added, and the mixture is reacted for several hours, the phosphorylated peptide turns into purple, thereby making it possible to identify the phosphorylated peptide. Further, streptoavidin labeled with a fluorescent pigment such as rhodamine is commercially available. Use of the streptoavidin labeled with the fluorescent pigment makes it possible to identify a phosphorylated peptide by a known fluorescent image analyzing method.

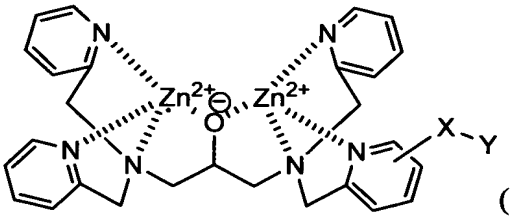
[0045]

It is possible to synthesize a compound equivalent to the compound (I), in which a methyl group or the like is introduced to a pyridine ring, to provide substantially the same operations and effects as in the embodiment of the present invention. Such a compound equivalent to the compound (I) is embraced in the scope of the present invention.

[0046]

The location of the (-X-Y) group in the inventive compound is not specifically limited. The (-X-Y) group may be located at the site as shown in the compound (I').

[0047]



(I')

[0048]

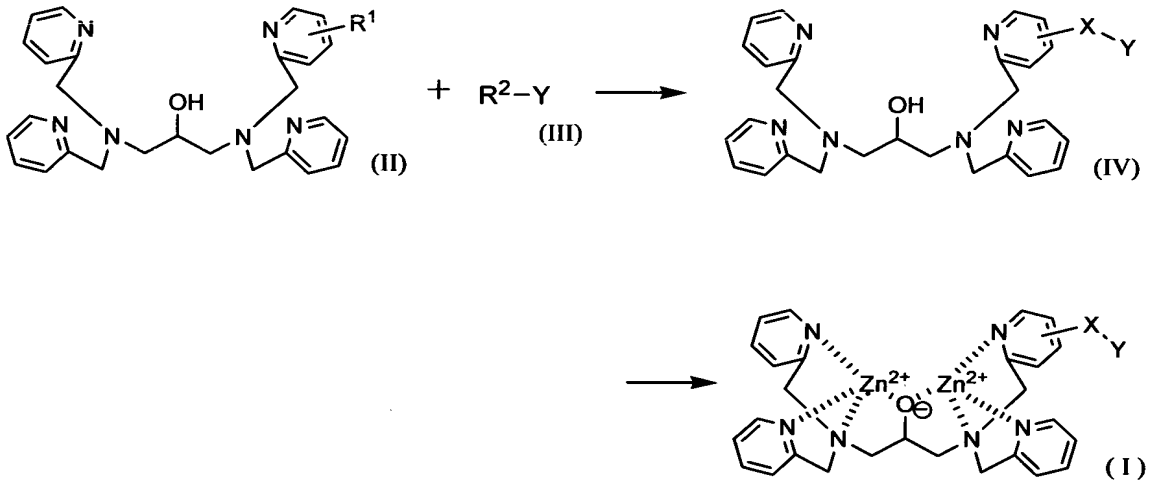
The compound (I) and the compound (I') are substantially equivalent to each other. Although it is not clear as to which compound is synthesized, the compound (I) or the compound (I'), what is actually synthesized is conceivably a synthesized mixture of the compound (I) and the compound (I'). It is needless to say that the compound (I') is embraced in the scope of the present invention.

[0049]

A complex compound represented by the formula (I) can be easily produced by a process comprising Scheme 1.

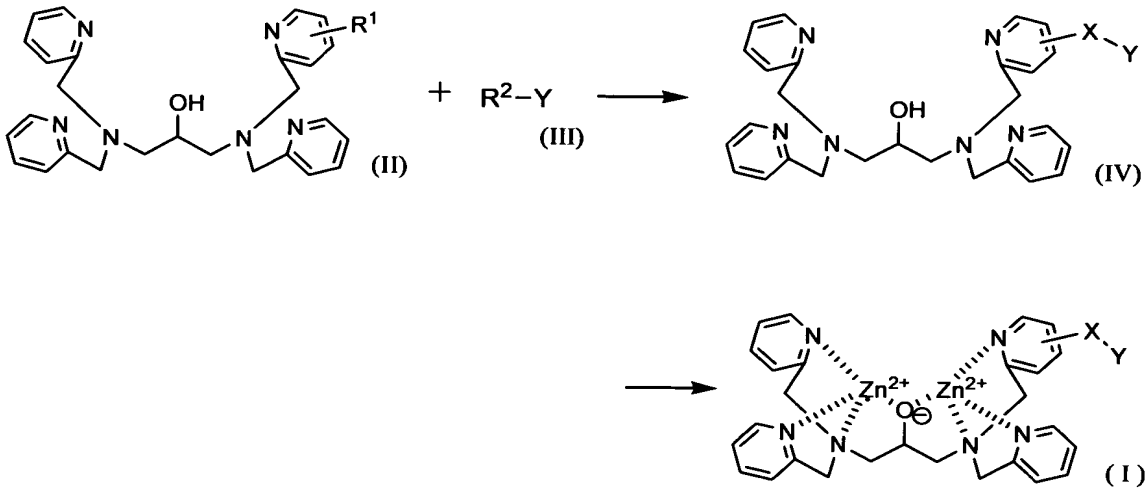
Scheme 1

[0050]



(II) (III) (IV) (I)

[0051]



wherein X and Y is the same as defined above, and R^1 and R^2 each is a reactive group for forming the linker moiety X.

In the above Scheme, the labeling group Y is introduced to the main skeleton via the linker moiety X by reacting the reactive groups R^1 and R^2.

[0052]

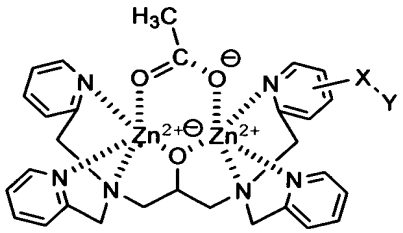
The kind of R^1, R^2, the solvent, a reaction temperature, a reagent other than the above, a purification method, and other factors are primarily determined by the kind of X. For instance, in case of introducing a labeling group via an amino group (a secondary or tertiary amino group), as a combination of R^1 and R^2, a combination of a group having an amino group (a primary amino group) at a distal end thereof and a elimination group such as a halogen atom. Condensing R^1 and R^2 under the presence of basic groups in the solvent is an example of general reaction condition. In case that R^1 is an active group, it is very easy to introduce a labeling group.

[0053]

Next, the compound (I) can be synthesized by adding a metallic salt to a solution containing the compound (IV). Zinc (II) nitrate or zinc (II) acetate may be added as the metallic salt. In case

that zinc (II) acetate is added, a compound represented by the following formula (V) in which acetic acid is temporarily coordinated is produced.

[0054]



(V)

[0055]

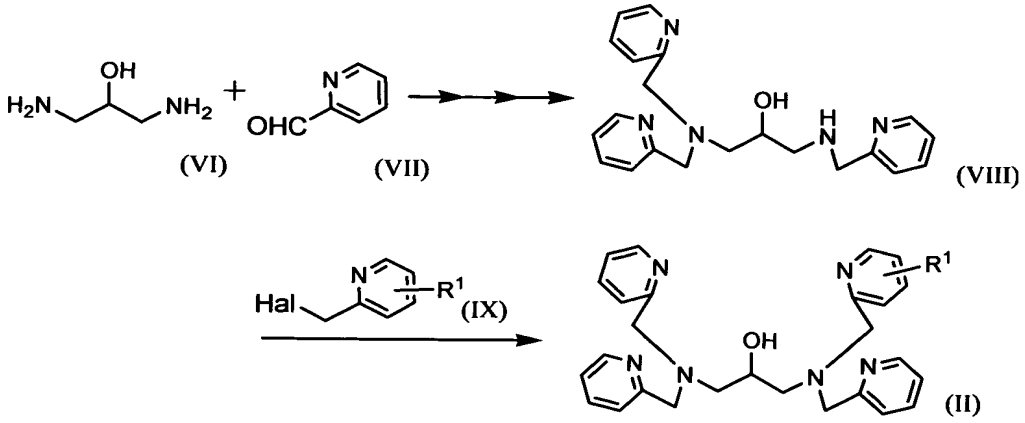
The compound (V) is chemically more stable than the compound (I), and accordingly useful in storage. The compound (V) is equivalent to the compound (I), and is usable in the similar manner as the compound (I). Specifically, by adding the compound (V) to the mixture containing the peptide, the phosphorylated peptide can be detected because the phosphoric monoester moiety is interchangeably coordinated to the compound (I) in place of the acetic acid.

[0056]

The compound (II), namely, a raw material compound of the compound (I), can be synthesized by the following Scheme 2.

Scheme 2

[0057]



(VI) (VII) (VIII) (IX) (II)

[0058]

wherein R^1 is the same as defined above, and "Hal" is a halogen atom, and preferably is a bromine.

The compound (VI), i.e., 1,3-diamino-2-propanol, as a raw material compound may be commercially available. Further, since both the compound (VII) and the compound (IX) have a relatively simple structure, the compound (VII) and (IX) may be commercially available, or can be synthesized by a well-known method for a person skilled in the art.

[0059]

In Scheme 2, first, the compound (VI) and the compound (VII) are reacted with each other under the presence of a catalyst for condensation to yield the compound (VIII). This reaction may be implemented step by step by introducing the compound (VII). Alternatively, the compound (VIII) can be obtained by a single step by using three or more equivalents of the compound (VII) to the compound (VI).

[0060]

In Scheme 2, reductive amination is carried out as a condensation reaction. A solvent used in the reductive amination is not specifically limited, as far as the solvent is capable of substantially dissolving the compound (VI) and the compound (VII), and does not inhibit the amination. For instance, alcohols such as methanol, ethanol and isopropanol; ethers such as diethyl ether, tetrahydrofuran and dioxane; water; or a mixed solvent containing two or more of these components can be used as the solvent.

[0061]

The reductive amination can be carried out with use of a conventional reducing reagent after condensing the compound (VI)

and the compound (VII) under the presence of concentrated hydrochloric acid, as a catalyst.

[0062]

An optimal condition regarding the reaction temperature and the reaction time can be optionally selected depending on the kind of the raw material compound or other factors. For example, the reaction may be carried out at a reaction temperature from 20 to 80 °C for a reaction time from 12 to 100 hours.

[0063]

After the reaction is completed, the solvent and the like are distilled off under depressurization before adding water. After water is added, the resultant mixture is extracted with a water-insoluble solvent, and the organic layer is dried over anhydrous magnesium sulfate or the like. Thereafter, the solvent is distilled off under depressurization. Subsequently, the residue is purified by a well known process such as silica gel column chromatography, thereby to yield the compound (VIII).

[0064]

The process for yielding the compound (VIII) is not limited to the process as shown by Scheme 2. Alternatively, the compound (VIII) may be synthesized using the compound (VI) and a halogen compound, for example.

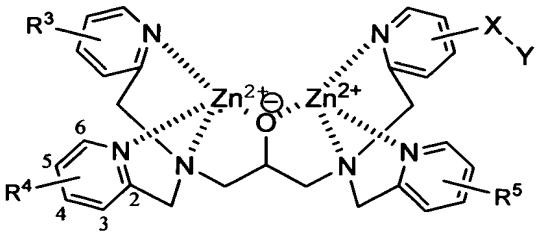
[0065]

Next, the compound (II) can be synthesized by reacting the compound (VIII) with the compound (IX). This reaction can be carried out by a known process of synthesizing tertiary amines.

[0066]

The following complex compound (X) can be used as a compound usable in the inventive method, in place of the complex compound (I).

[0067]



(X)

[0068]

wherein X, Y is the same as defined above, and R^3 through R^5 each is an electron donating substituent group at the 4 or 6 position on the pyridine ring.

The complex compound (X) used in the inventive method is electrically enriched with pyridine nitrogen by the electron donating substituent group that has been introduced to an appropriate position for substitution. Accordingly, the complex compound (X) used in the inventive method is highly coordinated to zinc, thereby making it possible to produce the complex compound (X) easily, while providing stability.

[0069]

A manner of using the complex compound (X), a process for producing the complex compound (X), and a raw material compound for the complex compound (X) are substantially the same as those regarding the complex compound (I).

[0070]

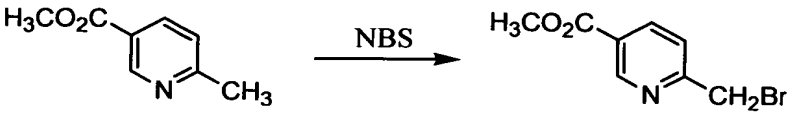
In the following, production examples and experiment examples are illustrated to describe the present invention in detail. The present invention is, however, not limited to the illustrated examples.

[0071]

[Examples]

(Production Example 1-1) Methyl 6-bromomethylnicotinate

[0072]



[0073]

To a solution of methyl 6-methylnicotinate (50g, 331mmol) in carbon tetrachloride (625mL), was added N-bromosuccinimide (59g, 331mmol). Further 1.0g of benzoyl peroxide was added, the mixture was reacted at a temperature from 40 to 50 °C for 24 hours with irradiation of light from a projector.

[0074]

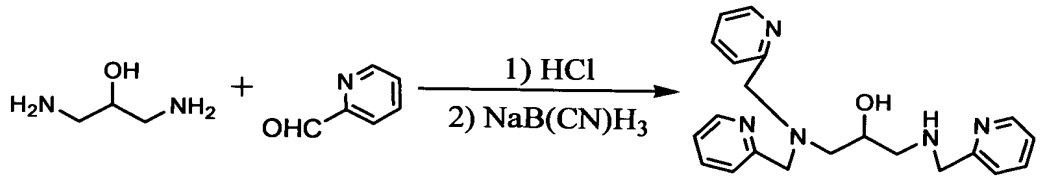
After the reaction mixture was cooled, the precipitated crystals were separated by filtration. The filtrate was washed with an aqueous solution containing sodium hydrogencarbonate, and concentrated. The residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 37g of the target compound.

^{1}H-NMR ($CDCl_3$, 300MHz) : δ 3.96(3H, s, OCH_3), 4.58(2H, s, CH_2Br), 7.54(1H, d, Py), 8.30(1H, dd, Py), 9.17(1H, d, Py)

[0075]

(Production Example 1-2) N,N,N'-Tri(2-pyridylmethyl)-1,3-diaminopropane-2-ol

[0076]



[0077]

To a solution of 1,3-diaminopropane-2-ol (32.6g, 362mmol) in methanol (2400mL), was added 60mL of concentrated hydrochloric acid. Further 2-pyridine aldehyde (116.3g, 1.09mol) was added dropwise,

and then sodium cyanoborohydride (50.16g, 798mmol) was added. After the addition was completed, the mixture was reacted at room temperature for 3 days.

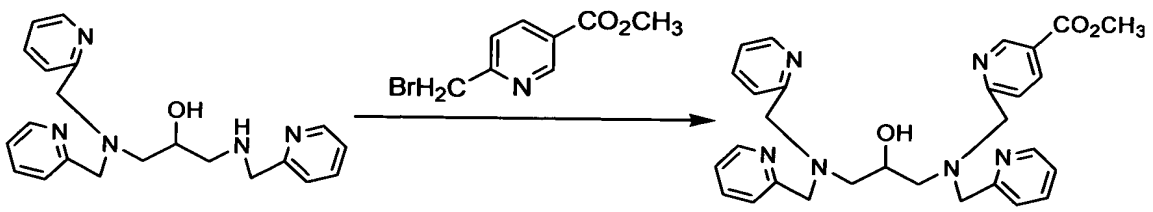
[0078]

After concentrated hydrochloric acid was added to the solution and the pH of the solution was adjusted to 6, the resulting solution was concentrated to some extent. Then, 0.1 N aqueous solution of sodium hydroxide was added to adjust the pH of the solution to 7, followed by extraction with chloroform. The extracts were collected and dried, the resultant was concentrated. The residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 34g of the target compound. ^{1}H-NMR ($CDCl_3$, 300MHz) : δ 2.59-2.83(4H, m, CH_2), 3.86-4.01(7H, m, NCH_2Py, CH), 7.15(3H, dd, Py), 7.23-7.32(3H, m, Py), 7.56-7.65(3H, m, Py), 8.53(3H, dd, Py)

[0079]

(Production Example 1-3) N,N,N'-Tri(2-pyridylmethyl)-N'-(5-methoxycarbonyl-2-pyridylmethyl)-1,3-diaminopropane-2-ol

[0080]



[0081]

To a solution of N,N,N'-tri(2-pyridylmethyl)-1,3-diaminopropane-2-ol (18.2g,

50mmol) obtained in Production Example 1-2 in dried dimethylformamide (150mL), was added potassium carbonate (13.8g, 100mmol), followed by addition of a solution of the methyl 6-bromomethylnicotinate (11.5g, 50mmol) obtained in Production Example 1-1 in dried dimethylformamide (75mL) dropwise. After the dropwise addition was completed, the mixture was reacted at 50 °C for 1 hour.

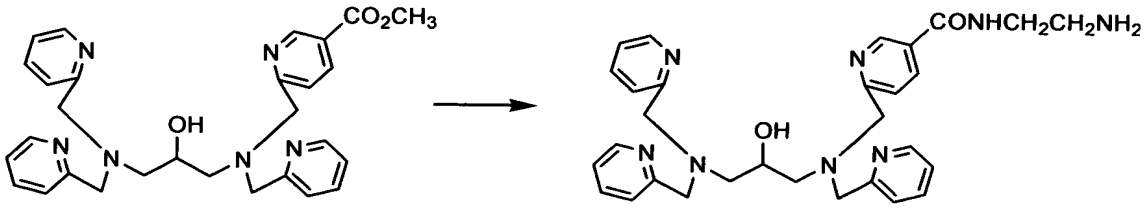
[0082]

After the reaction was completed, the solution was cooled. Then, the cooled solution was poured into 750 mL of water, and the pH of the solution was adjusted to 8 by adding 1 N hydrochloric acid. After extraction with ethyl acetate, the extracts were collected, washed with water and brine, and concentrated. The residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 21.5g of the target compound. ^{1}H-NMR ($CDCl_3$, 300MHz) : δ 2.58-2.73(4H, m, CH_2), 3.83-3.95(12H, m, OCH_3, NCH_2Py, CH), 7.10-7.14(3H, m, Py), 7.34(3H, d, Py), 7.50-4.60(4H, m, Py), 8.17(1H, dd, Py), 8.50(3H, d, Py), 9.09(1H, d, Py)

[0083]

(Production Example 1-4) N,N,N'-Tri(2-pyridylmethyl)-N'-[5-N"-(2-aminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol

[0084]



[0085]

To a solution of N,N,N'-tri(2-pyridylmethyl)-N'-(5-methoxycarbonyl-2-pyridylmethyl)-1,3-diaminopropane-2-ol (9.7g, 18.9mmol) obtained in Production Example 1-3 in methanol (100mL), was added ethylenediamine (22.7g, 378mmol) dropwise. After the dropwise addition, the mixture was reacted at room temperature for 3 days.

[0086]

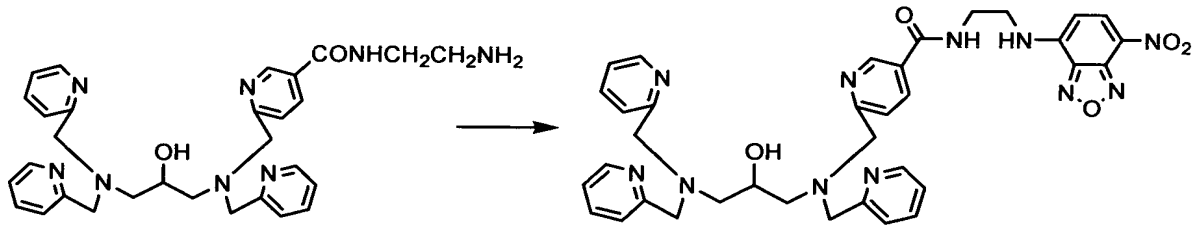
After the reaction was completed, the solution was concentrated, and the residue obtained by the concentration was purified by silica gel column chromatography, thereby to yield 9.72g of the target compound.

^{1}H-NMR ($CDCl_3$, 300MHz) : δ 2.54-2.71(4H, m, CH_2), 2.94(2H, t, CH_2N), 3.49(2H, dt, CH_2N), 3.80-3.99(9H, m, NCH_2Py, CH), 7.12(3H, ddd, Py), 7.35(3H, d, Py), 7.45(1H, d, Py) , 7.58(3H, ddd, Py), 8.02(1H, dd, Py), 8.49(3H, ddd, Py), 8.89(1H, d, Py)

[0087]

(Production Example 1-5)

[0088]



[0089]

To a solution of N,N,N'-tri(2-pyridylmethyl)-N'-[5-N"-(2-aminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol (200mg, 0.37mmol) obtained in Production Example 1-4 in acetonitrile (20mL), was added sodium hydrogencarbonate (336mg, 4.0mmol), followed by addition of

4-chloro-7-nitro-2,1,3-benzoxadiazole (73.8mg, 0.37mmol). The mixture was reacted at room temperature for 2 hours.

[0090]

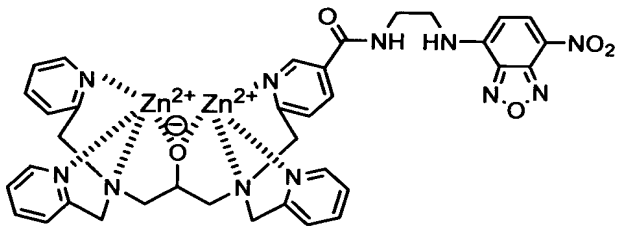
After the reaction was completed, the solution was concentrated. Then, 50mL of dichloromethane and 50mL of water were added, and the organic layer and aqueous layer were separated. The organic layer was dried over anhydrous sodium sulfate, concentrated, and the obtained residue was purified by silica gel column chromatography, thereby to yield 71.2mg of the target compound. ^{1}H-NMR ($CDCl_3$, 300MHz) : δ 2.46-2.69(4H, m, CH_2), 3.65-3.95(13H, m, NCH_2CH_2N, NCH_2Py, CH), 6.06(1H, d, Ar), 7.08-7.13(3H, m, Py), 7.32(3H, d, Py), 7.42(1H, d, Py) , 7.56(3H, ddd, Py), 7.96(1H, dd, Py), 8.44-8.48(3H, m, Py), 8.19(1H, d, Ar), 8.83(1H, d, Py)

[0091]

(Production Example 1-6) Solution containing the inventive zinc complex

[0092]

Compound of Production Example 1-5 →



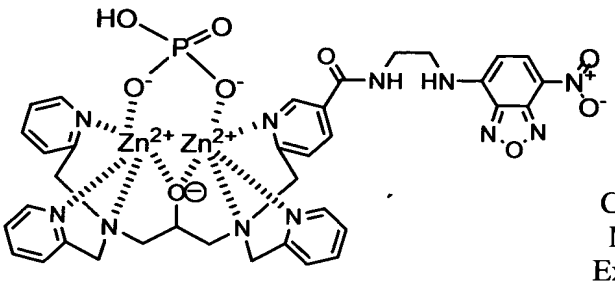
[0093]

Prepared was 50 μM aqueous solution containing the compound obtained in Production Example 1-5, followed by addition of zinc nitrate of 2 equivalents to the solution. Thus, a solution containing the inventive zinc complex was prepared.

[0094]

The zinc complex was identified according to the following method. Specifically, the compound obtained in Production Example 1-5 was dissolved in a phosphoric acid buffer (pH=6.86) to obtain the 50 μM solution, followed by addition of zinc nitrate of 2 equivalents to the solution. The inventive zinc complex in the solution exhibits the following structure, and was identified by MALDI-TOF mass spectrometer.

[0095]



$C_{36}H_{37}N_{11}O_9PZn_2$
Mol. Wt.: 929.49
Exact Mass: 926.11

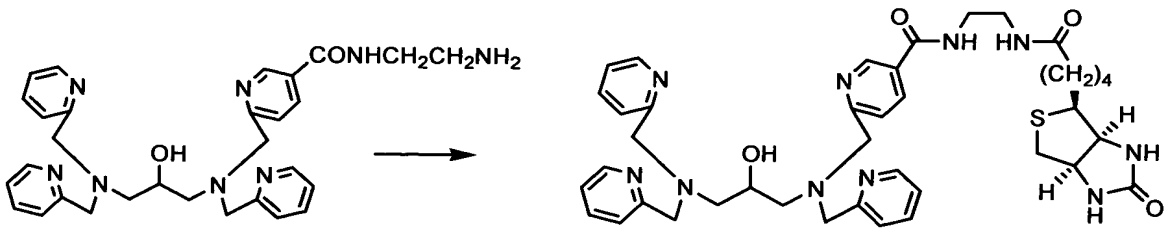
[0096]

The result of measurement by the MALDI-TOF mass spectrometer is shown in FIG.1. As shown in FIG.1, a molecular ion peak at 926.2 (exact mass: 926.11) was observed. It is considered that a molecular ion peak at 910.2 appeared because the oxygen in the oxadiazole group was eliminated.

[0097]

(Production Example 2-1)

[0098]



[0099]

To a solution of D-biotin (137mg, 0.56mmol) in dimethylformamide (10mL), was added 1,1'-carbonyldiimidazole (116mg, 0.72mmol). The mixture was reacted at room temperature for 12 hours. Thereafter, the solution was cooled with ice, followed by dropwise addition of a solution of N,N,N'-tri(2-pyridylmethyl)-N'-[5-N"-(2-aminoethyl)carbamoyl-2-pyridylmethyl]-1,3-diaminopropane-2-ol (260mg, 0.48mmol) obtained in Production Example 1-4 in dimethylformamide (3mL). The cooling bath was detached, and the reaction was carried out at room temperature for 2 hours.

[0100]

After the reaction was completed, the solution was poured into 50mL of water, followed by extraction with 50mL of chloroform twice. After the extracts were concentrated, the crude product was purified by silica gel column chromatography, thereby to yield 265mg of the target compound.

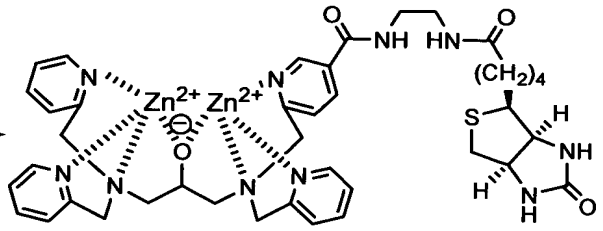
^{1}H-NMR ($CDCl_3$, 300MHz) : δ 1.27-1.47(2H, m, CH_2), 1.50-1.75(4H, m, CH_2), 2.13-2.26(2H, m, $COCH_2$), 2.52-2.74(5H, m, NCH_2, SCH_2), 2.79-2.88(1H, m, SCH_2), 3.01-3.12(1H, m, SCH), 3.43-3.65(4H, m, NCH_2CH_2N), 3.80-4.02(9H, m, NCH_2Py, CHO), 4.22-4.28(1H, m, NCH), 4.42-4.49(1H, m, NCH), 5.83(1H, bs, NHCO), 6.60(1H, bs, NHCO), 7.07-7.18(3H, m, Py), 7.31-7.38(3H, m, Py), 7.44(1H, d, Py), 7.59(3H, ddd, Py), 8.03(1H, dd, Py), 8.15(1H, bs, NHCO), 8.42-8.58(3H, m, Py), 8.94(1H, d, Py)

[0101]

(Production Example 2-2) Solution containing the inventive zinc complex

[0102]

Compound of Production Example 2-1 →



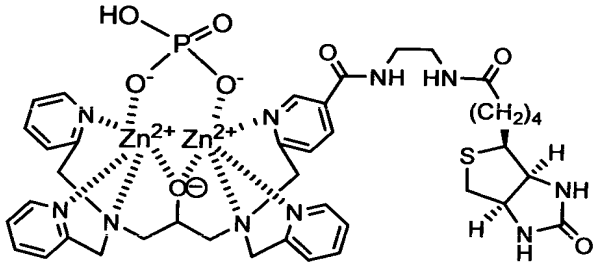
[0103]

The compound obtained in Production Example 2-1 was dissolved in a phosphoric acid buffer (pH=6.86) to obtain 3 mM solution, followed by addition of zinc nitrate of 2 equivalents to the solution.

[0104]

The inventive zinc complex in the solution exhibits the following chemical structure, and was identified by the MALDI-TOF mass spectrometer.

[0105]



$C_{40}H_{50}N_{10}O_8PSZn_2$
Mol. Wt.: 992.69
Exact Mass: 989.19

[0106]

A result of measurement by the MALDI-TOF mass spectrometer is shown in FIG.3. As shown in FIG.3, a molecular ion peak at 989.6 (exact mass: 989.19) is observed.

[0107]

Experimental Example 1: 10% Native-polyacrylic amide gel electrophoresis

First, prepared were gels for electrophoresis, a pH buffer for electrophoresis, and a coloring solution for dissolving samples

under the following conditions.

[0108]

Stacking gel:

125mM of Tris-hydrochloric acid buffer (pH=6.8)

4.5% (w/v) of polyacrylamide (acrylamide : bisacrylamide = 30:1)

Separation gel:

375mM of Tris-hydrochloric acid buffer (pH=8.8)

10% (w/v) of polyacrylamide (acrylamide : bisacrylamide = 30:1)

pH buffer for electrophoresis (pH=8.3):

25mM of Tris

190mM of glycine

Coloring solution for dissolving samples (3-times concentrated solution):

195mM of Tris-hydrochloric acid buffer (pH=6.8)

10% (w/v) of glycerol

0.1% (w/v) of bromo phenol blue (BPB), as a coloring marker used in electrophoresis

[0109]

Next, 2 μg each of 1: Bovine Serum Albumin, 2: β-casein (phosphorylated), and 3: β-casein (de-phosphorylated) was dissolved in the coloring solution to prepare samples. The respective samples were plotted on the gel. Then, a constant electric current of 4 mA was applied until the coloring marker was flowed out.

[0110]

The gel was immersed in the zinc-complex-containing solution (50 μM) obtained in Production Example 1-6 for about 30 minutes. Then, the gel was taken out from the solution, and photographed under

irradiation by a UV lamp. Further, the gel was dyed with Coomassie brilliant blue according to a conventional dying process, and the dyed gel was photographed. The gel dyed with the zinc-complex-containing solution is referred to as "gel A", and the gel dyed with Coomassie brilliant blue is referred to as "gel B", both of which are shown in FIG.2.

[0111]

As is obvious from FIG.2, according to the inventive method, β-casein being bonded with phosphoric acid can be identified exclusively. Thus, it is clear that the inventive method is advantageous in identifying phosphorylated peptides exclusively in samples derived from living organisms.

[0112]

[Effects of the Invention]

According to the inventive labeling method, the phosphorylated peptide, namely, a protein can be easily detected. Thus, the present invention is useful in diagnosing diseases or the like with use of samples derived from living organisms or the like.

[0113]

Further, since the inventive compound (I) shows a unique coordination bond to the two hydroxy groups in a phosphoric monoester moiety or phosphoric ion, the inventive compound (I) is useful as a compound usable in the inventive method. Additionally, the inventive compound (I) is useful for purifying or concentrating phosphorylated peptide and obtaining the chemical information of phosphorylated peptide.

[Brief Description Of The Drawings]

[FIG. 1] MALDI-TOF mass spectrum of a zinc complex according to the present invention.

[FIG. 2] Comparison of the results after electrophoresis.

[FIG. 3] MALDI-TOF mass spectrum of a zinc complex according to the present invention.

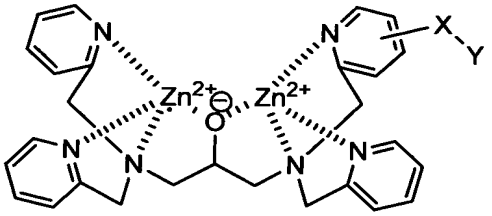
[Document] Abstract

[Object]

Provided are a method for easily detecting phosphorylated peptides, namely, proteins, in samples derived from living organisms or the like, and compounds that are highly coordinated to the phosphorylated peptides and usable in the methods.

[Method to Solve the Problem]

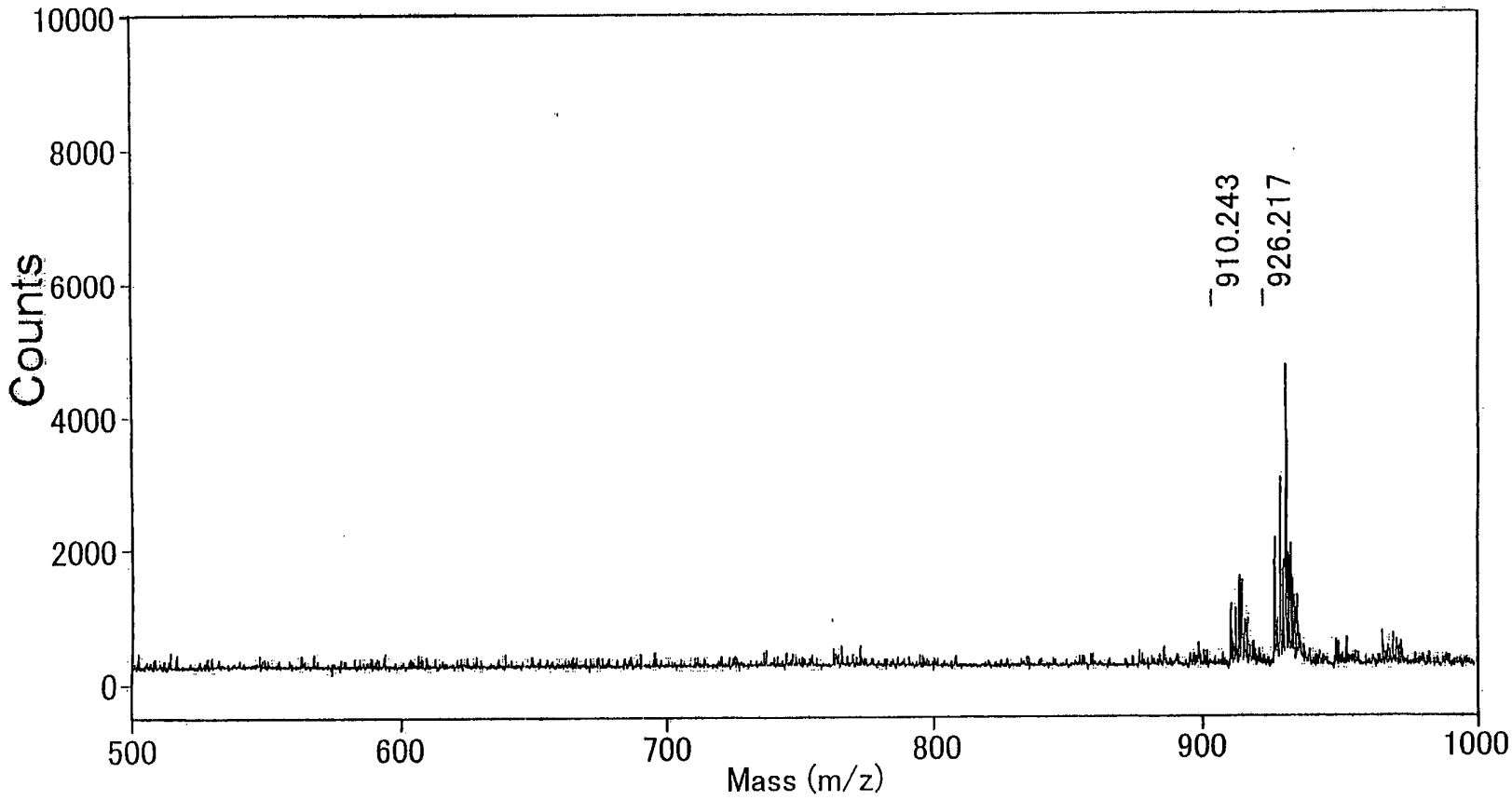
The complex compound is represented by the formula:



(I)

wherein X is a linker moiety, and Y is a labeling group. The compound (I) is highly coordinated to a phosphorylated peptide, and has a labeling group. Accordingly, with use of the compound (I), the phosphorylated peptide can be easily identified.

FIG.1



Comment: THAP posi ref

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	Guide Wire Voltage : 0.050 %	Pressure : 5.05e-07	Timed Ion Selector : 1276.0 OFF
	Delay : 50 ON	Low Mass Gate : 400.0	Negative Ions : OFF

FIG.2

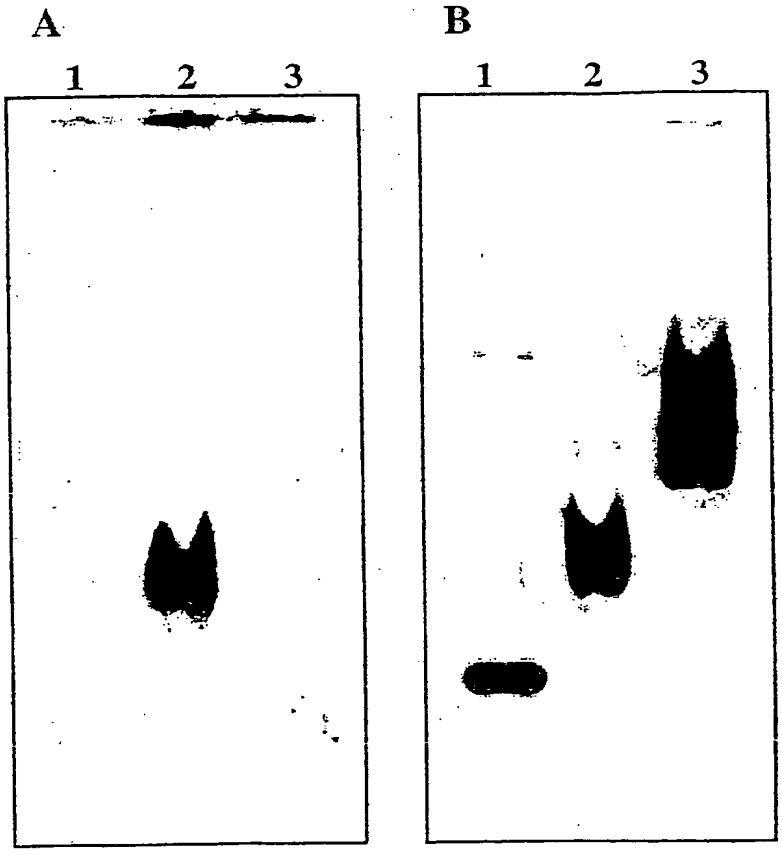
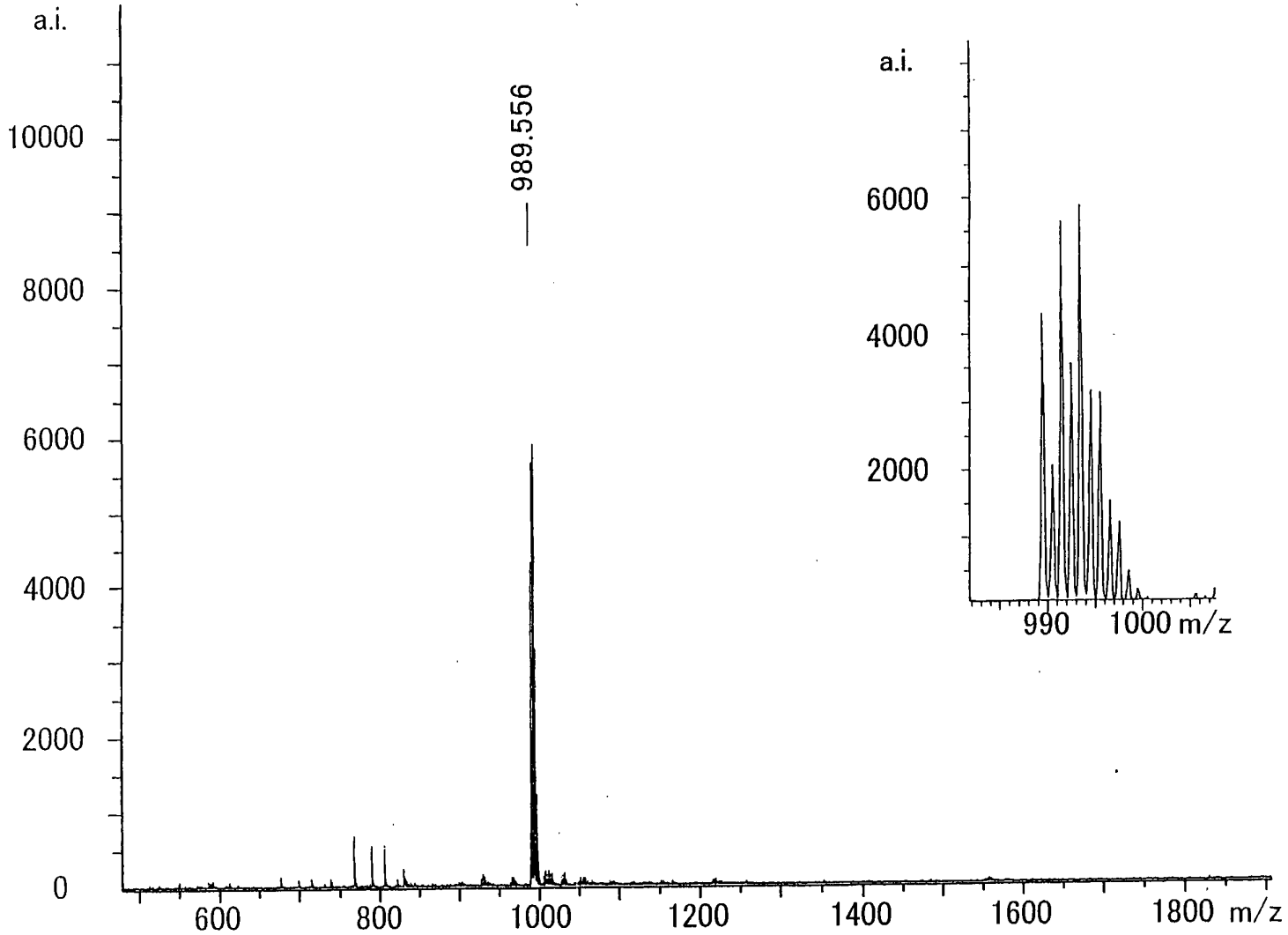


FIG.3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